

Micronutrients may influence the efficacy of ectomycorrhizas to support tree seedlings in a lowland African rain forest

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Abstract. In the lowland rain forest of SW Cameroon, a field experiment tested whether ectomycorrhizal hyphal connections might affect the growth and survival of seedlings of a principal tree species, *Microberlinia bisulcata*, close to its adults. Nursery-raised seedlings were planted into fine-, medium-, and coarse-mesh root bags, and as no-bag controls, in replicate subplots. The bags prevented fungal hyphae, and fine- and medium-sized roots, respectively, entering from the outside forest floor root mat. Harvests were taken after 1 and 2 yr, with non-destructive recording in between. Seedlings grew in typically low-light locations. Survivorship did not differ between treatments (33%), but seedlings grew significantly better in terms of stem dry mass by harvest 2 in the medium-mesh compared with other treatments. Treatment 1 to 3 seedlings had stem masses 25, 44, and 5% higher than controls, respectively. Using a method of differences across treatments, the positive effect of ectomycorrhizas on growth was 13.6%, while the negative effect of root competition (RCM) was 31.2% (net outcome = 17.6%). Adjustment was made to account for root penetration damaging some mesh bags. Differences in growth in replicate subplots were, however, much larger than those for treatments. Elemental analysis of seedling plant parts showed few differences between treatments, but phosphorus was high in stems, aluminum and iron were very high in roots, and copper was deficiently low in leaves. Soil analyses revealed very low copper levels, suggesting with the seedling results that this element was critically limiting for seedlings. Ectomycorrhizas are probably important for copper uptake (as for phosphorus), so roots may have been competing for this element. Because seedlings were growing in the shade and the soil was inhibitory to roots, they could not form network connections enough to positively out-balance root competition. The efficacy of ectomycorrhizal networks for at least seedling establishment in this forest is low.

Key words: copper as micronutrient; ectomycorrhizas; inhibitory soil factors; Korup National Park; lowland rain forest; rooting establishment; tree recruitment.

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INTRODUCTION

The possibility that adult and juvenile trees in closed forests might be functionally linked via ectomycorrhizal (ECM) networks has received considerable attention (e.g., Simard and Durrall 2004, Simard et al. 2012). There is some consensus that carbon and nutrients can probably move bi-directionally between some individuals via

fungus hyphal connections, but how large and significant are net transfers is still uncertain (Simard et al. 2002, Selosse et al. 2006). Whether the process operates generally to any important extent in any forest remains an open question: An interesting aspect is spatial and temporal variability in the movement of nutrients in relation to neighboring trees' physiologies. The appealing early idea was of a process by which

tree species might support their own, and even those of other, species' seedlings and saplings in the shade using ECM networks (Alexander 1989a, Courty et al. 2010). The simplest mechanism would be a passive one of nutrients moving along concentration gradients (Read et al. 1985). Other below-ground factors will have important effects on the growth of seedlings though, such as root competition, the relative demands of adults vs. juveniles for water and nutrients, and soil chemistry.

Several early demonstrations of nutrient transfer were made in highly controlled growth facilities using carbon and phosphorus tracers (Finlay and Read 1986a, b, Simard et al. 1997a, b), but support from the field has not been convincing. This led to doubts about whether direct transfer via the fungal mycelia was actually taking place, and alternative explanations offered were movement in the soil near to roots, conversion by microorganisms, or diffusion in root exudates (Newman 1988, Robinson and Fitter 1999). Transfer in the fungus might even only be as far as the recipient's mycorrhizal sheath and not into the plant itself in some cases (Wu et al. 2001). Showing that direct transfers do happen in the forest root layer has proved technically very challenging (see Hoeksema 2015).

Perry et al. (1992) have suggested that ectomycorrhizas may form the basis to group selection operating at the guild level in tree communities, forming complex adaptive systems in forests (Simard et al. 2012). However, arguing for a widely connecting fungal web at this level is contrary to the standard received theory of natural selection because some adults would be improving the recruitment of other species to the cost of their own fitness (Wilkinson 1998). The reason for the incompatibility of the proposed ideas, ecological and evolutionary, is that most ECMs are usually very general in their host associations (Alexander 1989b, Onguene and Kuyper 2001, Diedhiou et al. 2010, Ba et al. 2012, Peay et al. 2015). Linkages will occur between groups of neighbors of different species, even though the effective immediate transfers are probably more local. It may be more realistic to see the minor losses to heterospecific smaller neighbors as an inevitable negligible cost compared with potentially larger gains to conspecific ones, a form of connecting that is passively reciprocated among

different species in mixed stands (Simard and Durall 2004, Alexander and Lee 2005, Selosse et al. 2006).

Besides the evident nutrient acquisition trait, especially the ability of enhanced uptake of phosphorus, and possibly some micronutrients, from soils low in concentrations of these elements, ECMs may play a role in countering root pathogens, and even deter nematodes, but also they may be subject to invertebrate grazing (Smith and Read 1997). Field evidence for biotic interactions is still, however, very limited. Furthermore, the source-to-sink movement of carbon and other nutrients through a network might in some situations be the opposite of what is conventionally assumed, that is from juvenile to adult, when, for instance, the adult has a large internal carbon deficit due to old age (high respiration load; Newbery et al. 1998) and/or a heavy demand during a mast fruiting year (temporary high reallocation; Norghauer and Newbery 2016). If movement is bidirectional and fluctuates in the short term, net transfers could be zero and the linking process is then of little overall consequence for seedling growth and survival. Given that network linkage effects are likely to be small, idiosyncratic, and variable, other factors such as light levels, water availability, soil toxicity, and nutrient supply rates are going to be more important for seedling growth than any network transfers—at least when considering interactions between adults and seedlings. Being part of a network does not always mean that it has to be beneficial to the extent of supplying nutrients, but it could simply be a way for seedlings to become quickly and efficiently inoculated, and thereby gain their nutrients fairly independently from then on (Newman 1988, Newbery et al. 2000, Simard et al. 2002, Alexander and Lee 2005, Courty et al. 2010). Between any two (or more) neighboring adult trees, the amounts and rates of materials transported would be expected to be considerably larger, on the grounds of their very large combined biomasses, and hence carbon and nutrient equilibration is more feasible, for example, when a relatively smaller tree is more shaded than a larger one, or mast fruiting is occurring at different intensities among neighbors (Perry 1998, Norghauer and Newbery 2016).

Outcomes of experimental field studies have differed considerably in both results and

interpretations. In mixed temperate forests, the effect sizes were small and mostly insignificant, although for the family Pinaceae, for instance, occurring as it does in almost single-species stands, the evidence for seedling support by ECMs was reported to be stronger (Booth 2004, Kranabetter 2005, Teste and Simard 2008, Teste et al. 2009, Booth and Hoeksema 2010). An increasingly common method has been to use meshes of different gauge (aperture size) to selectively prevent hyphae and roots reaching seedlings from the surrounding forest network (e.g., Booth 2004, McGuire 2007, Teste et al. 2009). This involves setting seedlings, raised from seed ex situ or left to establish in situ, or even as transplants, inside rigid cylinders or flexible bags inserted in the ground. In all cases, outcomes were clearly also determined by environmental factors such as the level of light seedlings received, or whether the site was dry and water was a limiting factor. Distance between seedling and nearest adult tree stem has proven to be a particularly difficult variable to work with since it involves suites of factors operating in concert. Investigating the role of ECMs in the field is additionally beset with basically technical problems that arise from experimental manipulation of, and disturbances to, the ecosystem under study, these often creating serious artefacts that either obscure or bias the intended ECM treatments. Each study is therefore highly contingent on its setting-up and site conditions, and not just the species selected for study. Fully understanding what the treatments precisely involve (physically and biologically) is often lacking and therefore controversy follows. For example, first cutting holes in the ground severs roots and mycorrhizas around cylinders or bags, breaks the root mat, increases local root decomposition, and inevitably affects, even temporarily, how water and nutrients move in the soil inside and outside of cylinders and bags.

Most tests of the ECM hypothesis have been made in temperate forests, especially for tree species of interest to forest production, and contrastingly very few in the tropics for ecological research. The tropical ECM habit is often associated with monodominance, or codominance of closely related species, with trees growing often on very nutrient-poor sites, and where limited seed dispersal leads to seedlings establishing

mostly in the shade of conspecific adults (Connell and Lowman 1989, Hart et al. 1989, Torti et al. 2001, Peh et al. 2011, Corrales et al. 2018). By extension, it is of interest to test the general ECM network hypothesis for dominants which are not shade-tolerant as seedlings, yet have the other forest characteristics. ECM connections might contribute to supporting recruitment under more exacting physiological conditions where a seedling's own carbon supply from photosynthesis is a major limiting factor to growth. But, whether ECMs can effectively support seedlings that are already establishing poorly due to prior or contemporaneous factors, or only those that have become well established by the time connections can operate and be of benefit to them, is a key question.

In the tropics, there have been just three previous attempts to test ECM network effects on seedling growth and survival using exclusion techniques. Onguene and Kuyper (2002) planted *Paraberlinia bifoliolata* seedlings, either in closed PVC tubes or freely at different distances around single adults of this and three other species at Kribi, Cameroon, and found that the ones with full access grew much better. McGuire (2007) used a graded set of meshes to exclude hyphae and roots reaching *Dicymbe corymbosa* seedlings around adult trees of this species in a forest in Guiana, and found decreased growth and survival when ECM hyphae were excluded. Brearley et al. (2016) in a more extensive set of four experiments using mesh treatments too, in Bornean forest (Sabah), could find little evidence for ECM networks assisting seedling growth and survival of five dipterocarp species. In these three examples, the test (seedling) species were all shade-tolerant. *Dicymbe* and *Paraberlinia* grow in monodominant and codominant-mixed caesalpinaceae forests (Letouzey 1968), respectively, but *Shorea* species occur widely in very diverse forests, although the ectomycorrhizal habit is shared among them at the family level.

In southern Korup National Park, Cameroon, in rain forest groves of codominant caesalps, the most abundant and defining species in terms of tree basal area is *Microberlinia bisulcata* A. Chev. It occurs in large patches or groves which exhibit transient dominance (Newbery et al. 1998, 2004, 2013). This species has consistently very low and insufficient recruitment in situ, at least over the

last several decades, because seedling establishment is so very poor even though seeds and germinating seedlings are highly abundant after mast fruiting, which happens once every three years on average (Newbery et al. 2006a, Norghauer and Newbery 2015). Densities of saplings and of juvenile trees are also notably low, meaning that the groves are unlikely to replace themselves in the near future when the similarly aged and sized canopy trees die out (Newbery et al. 2010, 2013). In previous publications, different factors that might also explain the very low survival of seedlings near to adults have been reported: low light (Green and Newbery 2001a, b), nutrients (Newbery et al. 1997, 2002), and herbivores (Norghauer and Newbery 2013, 2014). Pathogens seem not to be implicated: Norghauer et al. (2010) using a fungicide trial found no evidence of them affecting *M. bisulcata* seedling survival. Previous experiments and demographic studies indicated that seedlings fared badly close to adult *M. bisulcata* trees especially, that is in the groves. This species and its codominants *Tetraberlinia bifoliolata* and *T. korupensis* are strongly ectomycorrhizal (Newbery et al. 1988). It appears then that being close to adults is of no saving benefit for small establishing *M. bisulcata* seedlings; perhaps, to the contrary, it is a distinct disadvantage, a feature which underlies this species life-history and ecology (Newbery et al. 2010). Nevertheless, without mycorrhizal connections, recruitment would perhaps be even more limited than it is. Given these conditions and background knowledge, it might be predicted that having ECM connections would be to no avail, although testing the general ECM network hypothesis for *M. bisulcata* might give new insights about how such a process balances out against others such as root competition, within the framework of the soil chemistry at Korup.

METHODS

Study site

The field experiment was carried out within the permanent 82.5-ha P-plot in southern Korup National Park, Ndian, Southwest Region of Cameroon (Newbery et al. 1998, 2004, 2009, 2013). The climate is strongly seasonal, with one pronounced dry season between December and February, and a long wet season, with

peak precipitation in August (annual rainfall 5116 ± 117 mm, 1984–2011; see also Newbery et al. 2006a). Soils are highly weathered, acidic, sandy, and well-draining (Gartlan et al. 1986, Newbery et al. 1997). The plot has been censused twice, in 1991 and 2005 (Newbery et al. 2013).

Design and setting up

The four blocks inside the P-plot selected for the 2011–2013 lysimeter study of Neba (2015) were used again because of their defined high abundances of adult *M. bisulcata* and correspondingly relatively low abundances of *T. bifoliolata* and *T. korupensis* trees (Appendix S1: Fig. S1). Within each block, three locations were selected, each approximately midway between an adjacent pair of large *M. bisulcata* trees. Averages of the two distances to nearest adults per location ranged from 8.2 to 19.0 m (mean and SE, 14.2 ± 0.9 m, $n = 12$; further details, and the calculation of inverse-distance weighted basal area estimates [wba], are given in Appendix S1: Table S1; refer also to Newbery et al. 2013 on tree spacing). This was close to being objectively at random as possible such that no adult tree was a neighbor of more than one location, and the pairs needed to be reasonably spaced out within a block to cater for within-block heterogeneity. At each location, a 4×4 m subplot was demarcated, orientated north, and divided into 16×1 m quadrats. Distance and direction to the large defining adults were recorded. The four treatments were the fine-root/ECM exclusion mesh sizes 0.5, 35, and 250 μ m plus a control (no mesh), each replicated four times; and the experimental design was a 4×4 Latin Square (LS). Nylon mesh bags (Plastok, Birkenhead, UK) had cylindrical dimensions 32 cm deep and diameter 16 cm. Each was closed flat-ended below, and the open top supported by a plastic ring. The 0.5- μ m mesh excluded ECM hyphae and rhizomorphs, and all roots; the 35- μ m one allowed hyphae and rhizomorphs to pass through; and the 250- μ m one allowed fine roots also to pass. Full access by seedlings to ECMs and roots of all sizes occurred under the control treatment.

Raising seedlings

Freshly fallen seed was collected at a smaller grove just outside the southern perimeter of the Park (Newbery et al. 2002), at the end of August

2013, and sown into beds in a shade house at the Mana Bridge nursery (see Neba et al. 2016). The best 280 most uniform seedlings of 450 germinants were potted as 14 batches of 20 each. Pots were of black polythene, 12 cm deep and 7.5 cm in diameter filled with the local sandy nursery oil palm plantation soil. Each pot received a few grams of fresh organic matter from under adult *M. bisulcata* trees in the P-grove to encourage ECM inoculation. Twelve batches were needed for the field experiment, two held in reserve. Seedlings were allocated at random to batches, with dying seedlings replaced from the extras within two weeks. Shading allowed 20–25% light transmission.

Nursery seedlings were allocated to treatments within batches, again at random, individually numbered with aluminum tags, and treatment-labeled with tape. All seedlings were in healthy condition with no visible signs of any diseases. On 13 September 2013, each was measured for its height (ht) and number of leaves (nlv). Height from the ground (first root) was done in two ways: to the cotyledon notch (ht1) and to the basal bracts of the uppermost leaf pair (unrelaxed, ht2). Numbers of small not-fully developed leaves and those flushing were noted. While 16 sound seedlings would be needed for the experiment proper, the other four of the 20 were kept for later as potential replacements. These sizes, at t_0 , would be used later as covariates of growth.

Field experiment

Seedlings were head-loaded in baskets, 12 km from the nursery to P-plot. Replicates of treatments were one more time allocated at random to their column and row positions within each Latin Square. At the center of each 1×1 m quadrat, a hole, ~30 cm deep and 20 cm diameter, was excavated with an auger, carefully placing the soil layer-by-layer onto plastic sheets. Occasionally, the hole needed to be slightly relocated by as much as 30 cm to avoid large stones or large roots. Small-to-medium roots were excised and disposed of: Stones > 2–5 cm were removed. The bag was inserted with its lip 2 cm above the soil surface, the soil was back-filled in reverse order until about half way, the seedling with its nursery pot soil positioned, and the rest of the excavated material lightly repacked around, with the surface

organic root mat replaced last. (This surface mat was unavoidably cut, and the returned material separated from outside the bag as a consequence.) For controls, a hole was dug in just the same manner as for treatments with bags, and the refilling and planting done likewise. The jettisoned smaller stones roughly matched the nursery pot medium in volume.

Seedlings received some stream water to assist root settlement. Out-planting was performed on 24 and 25 September 2013. In addition, six of the 12 subplots at random had seams of their mesh bag sealed with silicone, in a precautionary attempt to reinforce the manufacturer's sowing. The four additional seedlings per LS subplot stayed in their polythene pots, standing together at its center. These are referred to as standbys. Since there were fortunately no transportation mishaps, block 13 and 14 seedlings could remain in the nursery shade house for reference and comparison with the out-planted seedlings. On 13 November 2013, the 12 LS subplots were revisited and their 192 seedlings inspected. Any damaged or dead seedlings at this stage were replaced from among the four standbys. Those standbys not required for planting were maintained in order to have a further growth comparison. All seedlings were inspected on 4 January 2014 (t_1) and recorded for alive–dead status.

Recording and measurement

First complete non-destructive recordings of seedling growth were made on 25–27 June 2014 (t_2). Apart from registering any seedling deaths, survivors were measured for height (ht2) and number of leaves counted (nlv). There was no evidence of rodent clipping, nor of disturbances from larger animals like mandrills. Six LS subplots (hereon referred to generally as just subplots or, where individually, by the number prefixed by LS) were allotted to harvest 1 (H1) and six to harvest 2 (H2), with restricted randomization so that block pairs 1 + 2 and 3 + 4 would each have three subplots harvested per time. Fig. 1 illustrates two representative seedlings growing inside mesh bags. Harvest 1 was on 13 and 15 September 2014 (t_3). Second and third non-destructive harvests (t_4 and t_5) were made on 18 November 2014 and 19 May 2015 for those subplots remaining after H1, and a final H2 was taken on 3 November 2015. Bags were lifted by

carefully digging around them, the whole bag plus soil and plant placed in a large bucket of stream water, and the root system very gently eased from the soil. Bags were rinsed: Any invading roots from other trees were left inside. Control seedlings (no bag) were dug out to a depth of 30 cm. There were no cases of deeper rooting. Each seedling and its bag were kept together. The average time interval between planting out and H1 was 0.98 yr and that between H1 and H2 was 1.14 yr (Fig. 2).

After further washing, roots, stem, and leaves were separated, root length measured, roots



Fig. 1. Seedlings of *M. bisulcata* planted into mesh bags refilled with soil, within the experiment at Korup at the time of (a) the first (H1) and (b) the second (H2) harvests (photographs, DMN; 12 September 2014 and 5 November 2015). Note the fallen pod valves coming from neighboring adult trees of this species.

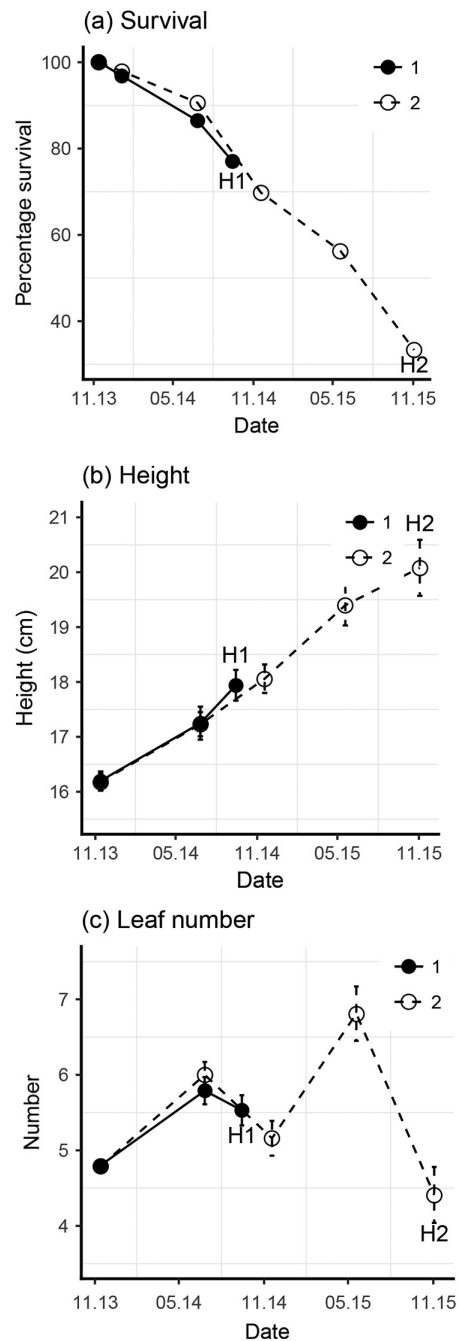


Fig. 2. Changes in number and mean sizes of *M. bisulcata* seedlings over the course of the experiment at Korup, at the times of non-destructive recording and harvesting (H1, H2): (a) survival, (b) height, and (c) leaf numbers of survivors. Those seedlings in the six subplots designated for H1 are shown as solid lines with closed circles and those six for H2 as dashed lines with open circles.

photographed (Appendix S2), stem length measured, and leaf number counted. Roots were scored for ECM infection at H1 (not H2): Generally, the off-white swellings were quite easily visible; otherwise, a hand-lens was required. A brown sheathing stem fungus occurred on just two seedlings at H1, and not at all at H2. It was easily removed from the outside of the infected stems. From visual inspection, no roots of any seedling, at either harvest, had any signs of damage or distortion from pathogens.

The state of each mesh bag was non-parametrically scored for root penetration as 1 = NRI (no foreign roots inside), 2 = RFO (roots from outside; subdivided into 2.0, low or few; 2.25, moderate or some; 2.5, many or large ones), 3 = CON (control, free access), and UNR, unrecorded (if the bag was damaged). The variable *rootpen1* used the coarse scores, and *rootpen2* included the finer divisions of score 2. Thus, status of the bag, its intactness, was partially confounded with treatment since all controls had *rootpen1* or *rootpen2* scores = 3.0 by definition. Several bags, especially the finer mesh ones, tore on extraction: For some others, there was evidence that cut root ends at the time of out-planting had led to a few tears. The three meshes ranged in tensile strength between 41 and 67 daN/mm² (manufacturer, *personal communication*): Bags showed no signs of deterioration due to the acid soils. During the course of the experiment, there was no evidence of waterlogging in any of the bags, particularly those with the finest mesh.

Plant parts were oven-dried at 65°C for 36 h and stored. Second and third non-destructive recordings were made on 18 November 2014 (*t*₄) and 19 May 2015 (*t*₅); and, for the six subplots left, H2 completed the experiment on 3 November 2015 (*t*₆), including harvesting the surviving standbys. Nursery batches 13 and 14 were each divided into two halves, with one set harvested at the same time as H1 and the other as H2.

The subplots were revisited on 31 May 2017 to collect soils. Around the perimeters of each of the still-relocatable 4 × 4 m areas used for the experiment, four (5 cm diameter) cores were taken. Loose organic material was brushed aside and samples sliced into three portions: for 0–2, 2–6, and 6–10 cm depth ranges. (Cores from last-to-be-sampled LS3 were not possible due to sudden extreme weather conditions.)

Elemental analyses

Dry masses of the harvested plant parts were determined by weighing. Leaf, stem, and root parts of replicates of treatments within subplots were separately bulked for H1 because plants at that date were generally too small to provide sufficient material for chemical analysis (24 samples each part; 4 treatments × 6 subplots). At H2, while there was sufficient material for each surviving replicate to be analyzed separately (32 samples), for leaves there was even less available than at H1. This meant bulking replicates for subplot 5, and those for subplot 11; but bulking those of subplots 2, 4, and 12 all together as one pseudo-subplot 15 (12 samples; 4 treatments × 3 subplots). At H2, LS9 had no survivors. After redrying, samples were milled, and 300 mg of each was digested in a 2.5-mL mixture of Se, sulfuric acid, and salicylic acid and analyzed for total Ca, K, Mg, Al, Fe, Mn, B, Cu, and Zn using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES; model Optima 7000DV; Perkin-Elmer, Waltham, Massachusetts, USA), and total N and P on a Continuous Flow Optical Absorption Spectrometer (CF-OAP; model Skalar Scan+; Skalar Analytical, Breda, The Netherlands). Soil samples were air-dried, extracted in 1 mol/L KCl, and analyzed for Al, Fe, Mn, Cu, Zn, and Co on the ICP-OES.

Statistical analysis

Analyses of variance and general linear models, particularly those for unbalanced designs using restricted maximum likelihood (REML) estimation, were run using GenStat version 18 [2015] (Payne et al. 2011). The analysis of the Latin Squares repeated across blocks is discussed by John (1971). Graphics were produced with ggplot2 in R (Wickham 2016).

RESULTS

Survival and seedling mass

The rate of mortality of out-planted *M. bisulcata* seedlings across the almost 2 yr of the experiment was nearly constant, with numbers of survivors declining linearly over time (Fig. 2a). The two sets of blocks which were destined for H1 and H2 had very similar losses in numbers by the time of H1 (survival ~78%). By H2, survival was 33% (32/96 seedlings). GLMM analysis,

using simply replicates within subplots (binomial error, logit link), revealed that survival did not differ significantly between treatments, at either H1 or H2 (quasi- $F = 1.35$, $P = 0.262$; quasi- $F = 0.91$, $P = 0.438$; resp., $df = 3, 87$).

Dry masses of leaf, root, and stem separately, and these combined as total surviving seedling mass, were subject to unbalanced analysis of variance, which for H1 accounted for row and column effects of the Latin Square arrangements within each subplot. The lack of balance across subplots and treatments was due to seedling mortality. For leaf and total masses, there were 74 values (the number of seedlings alive); for stem and root mass, 11 recently dead leafless seedlings were included to provide 85 values. By H2, too many seedlings had died to permit fitting of LS column and row terms, reducing effectively to a randomized block, that is, subplot, design. One subplot even had no survivors. The numbers of values for leaf and total, and for stem and root, mass at H2 were 60 and 66, respectively. Each analysis was rerun to include the following covariates: either initial ht1, ht2, or lvn.

At H1, treatments were significant in some cases (Table 1; $P \leq 0.05$): (1) for leaf mass (with ht2 as covariate) at $P = 0.013$ and (2) for total mass (with ht1 as covariate) at $P = 0.024$ (with ht2 as covariate, $P = 0.031$). In addition, at H1, root mass was marginally significant but rather widely at $P = 0.099$. Control and treatment 2 leaf/total masses did not differ from one another (+2.5% increase over control), but mass under treatment 1 was more (+6.8%), and under treatment 3 the most (+21.3%). Most of this latter increase was due to leaf mass under treatment 3 being 35.9% over the control (Table 1). At H2, stem mass was marginally significant, with covariate ht2, at $P = 0.054$ (Table 1); and leaf mass less so at $P = 0.098$. The effect of the covariate (ht2) was marginally significant for leaf mass (H1), but more significant for stem (H1, H2), root (H1), and total (H1) mass ($P < 0.02$). At H2, for the most robust outcome, seedlings under treatments 1, 2, and 3 (the mesh bags) had, respectively, 25.1, 43.7, and 4.5% greater stem mass than those growing as controls (Table 1). These treatment differences using all bags are analyzed again later, after making allowances for any bias caused by roots penetrating the bags. Thus, between H1 and H2, the

strongest effects switched from under treatment 3 (for leaf mass) to under treatment 2 (for stem mass), not a reinforcement of one treatment effect over time.

Between H1 and H2, overall simple mean leaf mass per seedling, irrespective of subplot and treatment and unadjusted for covariates, decreased by 53.7% (H1, 0.205 ± 0.010 ; H2, 0.095 ± 0.009 g; $n = 74, 32$), stem mass slightly increased by 6.9% (H1, 0.216 ± 0.008 ; H2, 0.231 ± 0.013 g, $n = 85, 32$), root mass was unchanged (H1 and H2 both 0.158 ± 0.008 g; $n = 85, 32$), and total mass decreased by 20.2% (H1, 0.605 ± 0.021 ; H2, 0.483 ± 0.025 g, $n = 74, 32$). In addition, root length was almost constant (H1, 12.82 ± 0.42 ; H2, 12.63 ± 0.45 cm, $n = 85, 32$). The decline in leaf mass (and number) is not readily explained by seasonal differences because H1 and H2 were at similar dates in 2014 and 2015.

Variance removed by subplots (as blocking), using all bags, was significant for stem, root, and total mass ($P \leq 0.02$), particularly for root mass ($P < 0.001$). The efficiency of the Latin Square was shown especially by the significance of subplot.row (not always, however, at right angles to the line between the adults) and again for root mass ($P = 0.005$), although interestingly not for subplot.col ($P = 0.35$)—apart from total mass for rows ($P = 0.004$), other row and column effects for mass were not significant. When allowed bags were analyzed, subplot effect was significant ($P \leq 0.05$) for all mass variables, strongest for root mass ($P = 0.003$), but now the subplot.row factor was significant too for all mass variables ($P \leq 0.05$). Notably, the variable most affected by between- and within-subplot heterogeneity was root, and not leaf or stem, mass.

Mycorrhizal status

Considering all seedlings at H1 ($n = 96$), 85 had roots which could be scored for ECM (a few had lost leaves shortly before). Overall, 25 (29.4%) had visible ECMs, but between treatments, only 1/20 (5%) had ECMs under the control (treatment 4), 7/16 (43.8%) with the 250- μ m mesh (3), 7/20 (28%) with the 35- μ m one (2), and finally 10/22 (45.5%) for the finest 0.5- μ m mesh (1). The presence of ECMs was significantly associated with treatments (likelihood ratio $\chi^2 = 10.56$, $df = 3$, $P = 0.014$). Unexpectedly

Table 1. Mean dry mass (g) of plant parts (L, leaf; S, stem; R, root) and their totals (T) for *M. bisulcata* seedlings at harvests 1 and 2 (H1 and H2), grown in all of the bags of four different mesh sizes within forest subplots.

Harvest	Plant part	Treatments				F-ratios	
		1 (0.5 μ m)	2 (35 μ m)	3 (250 μ m)	4 (control)	Treatments†,‡	Covariate
H1	L	0.206 ^{ab}	0.172 ^b	0.246 ^a	0.181 ^b	4.23*	3.89 [§]
	S	0.221 ^a	0.216 ^a	0.226 ^a	0.189 ^a	1.08 ^{ns}	10.38**
	R	0.150 ^a	0.154 ^a	0.182 ^a	0.155 ^a	2.24 [§]	6.79*
	T	0.597 ^{ab}	0.573 ^b	0.678 ^a	0.559 ^b	3.40*	12.54***
H2	L	0.080 ^a	0.128 ^a	0.120 ^a	0.073 ^a	2.36 [§]	0.03 ^{ns}
	S	0.249 ^{ab}	0.286 ^a	0.208 ^b	0.199 ^b	2.95 [§]	9.27**
	R	0.155 ^a	0.165 ^a	0.159 ^a	0.154 ^a	0.09 ^{ns}	1.16 ^{ns}
	T	0.483 ^a	0.579 ^a	0.487 ^a	0.426 ^a	1.70 ^{ns}	3.62 [§]

Notes: Treatment means are adjusted for the covariate ht2. Means that do not share any same small letters are significantly different at $P \leq 0.05$.

*** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; $^{\S}P \leq 0.10$, $^{ns}P > 0.10$.

† H1: df treatments = 3, 29 (Leaf and Total), or 3, 39 (Stem and Root); df covariate = 1, 29 or 1, 39.

‡ H2: corresponding df = 3, 23; and 1, 23, all plant parts.

then, the controls with no mesh bags were very infrequently infected, those letting small roots and hyphae through a little more, but the most were for the treatment which was expected to completely exclude hyphae from the outside. Logistic regression indicated that the difference between treatments 1 and 4 was the most significant of all comparisons ($t = -2.486$, $df = 81$, $P < 0.01$). Subplots, however, did not differ significantly in their proportions of seedlings infected ($\chi^2 = 7.52$, $df = 5$, $P = 0.185$).

Root penetration

Analyses of variance were repeated by taking into account the extent of root penetration which to different degrees might have compromised the treatments. Instead of retaining simply the intact bags (bags with no root entry at all), different mesh sizes, by design letting some roots in, led to the following criteria. For treatment 1 (0.5- μ m mesh), no bags with any root entry at all were allowed (rootpen2-values of 2.0, 2.25, and 2.5), and this excluded $n = 6$ seedlings. For treatment 2 (35- μ m mesh), bags with values of 2.0 ($n = 1$) were allowed but not those with 2.25 and 2.5 ($n = 3$). Lastly, for treatment 3 (250 μ m), bags with values of 2.0 and 2.25 ($n = 9$), but not 2.5 ($n = 6$), were allowed. All rootpen2-values scored UNR ($n = 8$ overall) were additionally not allowed since these torn bags may well have had roots which were left in the soil on bag extraction, and their status was indeterminate. Thus, instead

of the 17, 15, and 7 seedlings for treatments 1, 2, and 3 when only intact bags were retained, the graded allowance resulted in 17, 16, and 16 replicates, thereby maintaining a still reasonable sample sizes but not allowing unintended root penetration to unduly bias the treatment comparisons. The 24 control seedlings were all allowed.

At H2, the same considerations allowed 17, 24, and 16 seedlings for the treatments 1, 2, and 3 (instead of 17, 19, and 2 where bags were fully intact). Not all bags at H2, nor at H1, would have had live seedlings, so the corresponding numbers per treatment for dry masses were lower. The numbers of allowed bags, using the criteria applied for H1, were $n = 4$, 0, and 4 for treatments 1 to 3, respectively. As expected, by the time of H2, bags with the largest mesh size had let in many small roots from the outside. Applying silicone or not to the seams of mesh bags (subplot-level) was not significantly associated with rootpen2 (scores 2, 2.25, and 2.5) at either H1 (likelihood ratio [LR]- $\chi^2 = 3.14$, $df = 2$, $P = 0.21$) or H2 (LR- $\chi^2 = 3.11$, $df = 2$, $P = 0.21$). At H1, the numbers of cases of rootpen1 = 2 for with vs. without silicone were 11 and 14 and at H2 18 and 15, respectively. In the course of harvesting, there was no visual evidence of any activity by invertebrate animals within the bags, and none around the control seedlings.

Despite these careful considerations, outcomes were quite similar to using all bags intact or damaged. The significance of treatment as a

factor decreased slightly at H1 (Table 2) with now $P = 0.030$ for leaf mass, and $P = 0.033$ for total mass, and marginally for stem mass at $P = 0.68$. At H2, the effect on stem mass became more significant at $P = 0.019$, with leaf and total mass remaining weakly significant at $P = 0.86$ and 0.75 , respectively. For allowed seedlings at H1, the pattern was very similar to all, with differences compared with control being ~ 0 , $+8.2$, and $+16.5\%$, respectively (Table 2). Again, at H2, seedlings under treatments 1, 2, and 3 had stem masses, respectively, 26.2 , 47.2 , and 18.5% greater than controls, increases which were slightly higher compared with all bags for 1 and 2 but much higher now for 3 (Table 2). At H1, leaf mass was better correlated with stem mass ($r = 0.701$, $df = 72$, $P < 0.001$) than with root mass ($r = 0.454$, $df = 72$, $P < 0.001$), while the correlation between stem mass and root mass was intermediate ($r = 0.579$, $df = 83$, $P < 0.001$). At H2, the respective correlations were similar to those at H1 ($r = 0.564$, 0.336 , and 0.593 ; $df = 30$, $P < 0.001$). This meant that, firstly, where cases of root invasion may have violated some treatments, they had rather weak consequences, and secondly, the differences were being confirmed (importantly for stem mass at H2) under the more conservative and precise testing that used allowed seedlings.

Root invasion that came into the bags unsolicited might have provided an explanation for the lack of strong differences between treatments. But again for all ECM-scored roots ($n =$

81, with 4 of the 85 missing), overall 24 (29.6%) were infected. Putting the rootpen2-scores 2.0, 2.25, and 2.5 back into one group 2 (as rootpen1) because of small sample sizes, in the cases of no penetration at all (intact, level 1) 16/36 (44.4%) were ECM, and if roots did come in (level 2) 7/25 (28%)—with the 1/20 (5%) for controls, as before. The association was significant (LR ratio $\chi^2 = 11.40$, $df = 2$, $P = 0.003$). Leaving aside the controls, intact and penetrated bags had 16/36 (44%) and 18/25 (72%) of seedlings were infected, respectively, but not differing significantly ($\chi^2 = 1.728$, $df = 1$, $P = 0.189$). Limiting even further to just treatments 2 and 3, those that should have allowed hyphal connections via meshes, the corresponding proportions were 8/21 (38.1%) and 5/19 (26.3%) infected—an even less significant outcome ($\chi^2 = 0.636$, $df = 1$, $P = 0.425$). Allowed seedlings with rootpen1 = 2 had 3/10 (30%) infected (cf. the 44% for intact). Root penetration therefore did not appear to affect ECM infection, a notable result too.

Non-destructive measurements

Starting heights (ht2) of the seedlings, before out-planting (t_0), destined for H1 and for H2, were not significantly different between treatments ($F = 0.23$, $df = 3, 51$, $P = 0.87$; and $F = 0.01$, $df = 3, 15$, $P = 0.999$, respectively) giving reassurance of similarity in average plant size allocated across the experiment. Treatments did not differ significantly in height at either at t_2 or t_3 for H1 seedlings ($P = 0.16$ – 0.49), and nor for

Table 2. Mean dry mass (g) of plant parts (L, leaf; S, stem; R, root) and their totals (T) for *M. bisulcata* seedlings at harvests 1 and 2 (H1 and H2), grown in the allowed bags of four different mesh sizes within forest plots.

Harvest	Plant part	Treatments				F-ratios	
		1 (0.5 μm)	2 (35 μm)	3 (250 μm)	4 (control)	Treatments ^{†,‡}	Covariate
H1	L	0.203 ^{ab}	0.171 ^b	0.234 ^a	0.181 ^b	3.92*	7.29*
	S	0.240 ^a	0.220 ^a	0.224 ^a	0.203 ^a	2.77 [§]	8.89**
	R	0.158 ^a	0.157 ^a	0.165 ^a	0.158 ^a	0.87 ^{ns}	10.92**
	T	0.605 ^{ab}	0.557 ^b	0.651 ^a	0.559 ^b	3.80*	18.62***
H2	L	0.090 ^a	0.127 ^a	0.116 ^a	0.071 ^b	2.63 [§]	0.00 ^{ns}
	S	0.246 ^{ab}	0.287 ^a	0.231 ^{ab}	0.195 ^b	4.44*	7.04*
	R	0.136 ^a	0.164 ^a	0.183 ^a	0.154 ^a	0.54 ^{ns}	0.26 ^{ns}
	T	0.472 ^a	0.579 ^a	0.529 ^a	0.420 ^a	2.78 [§]	2.12 ^{ns}

Notes: Treatment means are adjusted for the covariate ht2. Means that do not share any same small letters are significantly different at $P \leq 0.05$.

*** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; $^{\S}P \leq 0.10$, $^{ns}P > 0.10$.

[†] H1: df treatments = 3, 15; df covariate = 1, 15, all plant parts.

[‡] H2: corresponding $df = 3, 16$; and 1, 16, all plant parts.

H2 seedling height at t_2 , t_4 , t_5 , and t_6 were there any significant differences ($P = 0.61\text{--}0.75$). Number of leaves (lvn), like ht2, did not differ at the start (t_0) for H1 ($F = 0.57$, $df = 3, 51$, $P = 0.64$) and H2 seedlings ($F = 0.08$, $df = 3, 51$, $P = 0.97$). And, for neither set of seedlings were there differences at t_2 , t_4 , t_5 , and t_6 ($P = 0.383\text{--}1.00$). At the time of harvesting of H1 (t_3) and H2 (t_7), none of height (ht2), leaf number (lvn), or root length (rln) differed between treatments ($P = 0.14\text{--}0.85$), and with the allowed data the same ($P = 0.16\text{--}0.94$). Height increment, on average, was very small, indicating slow growth, at ~ 4 cm gain in 2 yr: Leaf number fluctuated as result of seasonal turnover (Fig. 2b, c). The reader is referred to the photographs in Appendix S2 for impressions of root form and architecture in the LS experiment, and in comparison with the seedlings remaining in the nursery. The LS seedlings' roots shown are a randomly mixed selection across the treatments.

Neighboring abundance of adult trees

Inverse-distance weighted basal area estimates (wba) were evenly distributed across blocks (mean and SE: 10.0 ± 0.8 dm²/m, $F = 0.39$, $df = 3, 8$, $P = 0.76$). Mean wba for H1 was only slightly greater than for H2 (10.88 ± 1.50 vs. 9.16 ± 0.74 dm²/m; $t = 1.04$, $df = 5$, $P = 0.33$). These tests affirm effective replication at the block level, and a lack of temporal bias caused by any difference in wba across harvests.

Dry masses of leaves, stems, and roots, and their totals, and height, number of leaves, and root length (all bags), were correlated for each treatment separately against mean per subplot at H1 ($n = 6$), showing highly significant positive correlations for stem, root, and total masses, and likewise root length, under treatment 1 (fine-mesh bags; $r = 0.985$, $P < 0.001$; $r = 0.939$, $P = 0.005$; $r = 0.913$, $P = 0.011$; and $r = 0.832$, $P = 0.040$; respectively). No other combination of response variable and treatment was significant at $P \leq 0.05$, except for root length under treatment 3 (course-mesh bags; $r = 0.989$, $P < 0.001$). This seems to indicate that seedlings in subplots with high nearest-neighbor adult *M. bisulcata* abundances had better growth than those with low abundances, when ECM hyphae but not fine roots were allowed to enter bags from the outside. At H1, the difference in basal area was

almost twofold between the three high and three low abundance subplots (see Appendix S1: Table S1), and this was matched by significant differences in stem and root mass, and root length ($t = -17.82$, $P = 0.003$; $t = -3.46$, $P = 0.41$; $t = -3.63$, $P = 0.036$; respectively), but not in total mass ($t = -2.90$, $P = 0.10$). At H2, no correlations between basal area and the response variables under the four treatments were significant at $P \leq 0.05$, suggesting further that the nearest-neighbor influence disappeared after the first year of seedling establishment in the forest.

Even so, when only allowed bags were used in a similar analysis for stem, root, and total masses, and likewise root length, under treatment 1 at H1, correlations were still positive yet statistically marginally significant ($r = 0.627$, $P = 0.18$; $r = 0.747$, $P = 0.088$; $r = 0.70$, $P = 0.051$; and $r = 0.721$, $P = 0.11$; respectively). In addition, root mass and root length again were correlated with basal area under treatment 3 ($r = 0.782$, $P = 0.066$; $r = 0.7609$, $P = 0.079$). The strongest correlations were for the means of treatment responses, for root mass and root length ($r = 0.810$, $P = 0.051$; $r = 0.965$, $P = 0.002$). Differences in these variables between the two basal levels were, however, no longer significant ($P \geq 0.17$). At H2, as at H1, there were no significant correlations, in part due to the now, much lower, treatment replication for survivors; and only five subplots had seedlings. The more conservative analysis, by ensuring that root penetration into the finer mesh bags was not compromising intended treatment effects, implies that root competition was indeed probably overriding any ECM benefits to seedlings at H1. The increased seedling root growth when outside fine roots were allowed may support the argument that the latter carry ECM hyphae to the former.

Calculation of RCM and ECM effects

Working on the assumption that the intensity of root competition was the same for the fine- and medium-mesh treatments, and taking medium-mesh treatment as a reference, the negative effect of root competition (RCM) is the difference control-medium, and the positive effect of a network (ECM) is the difference medium-fine, of shoot dry mass at H2. That controls were very little infected by ectomycorrhizas means that root

competition and ECM connection effects were not confounded within this control treatment. The simple calculations assume an additivity in the two effects across treatments.

At H1, RCM and ECM effects were small and within $\pm 12.5\%$ (Table 3), with an average of all and allowed bags analyses showing 10.1% for RCM and -5.7% for ECM, that is, ECM was also apparently negative in effect on seedlings at this stage. But by H2, the differences had increased considerably, with averages of 31.2% for RCM and 13.6% for ECM, a 2.3-fold difference indicating that root competition was much larger a negative effect than was the putative ectomycorrhizal linkage a positive one on seedling growth. Considering, difference in stem mass between H1 and H2, that is, absolute growth, effects showed up even more strongly, although with less difference: Averaging again over the two analyses, the RCM effect was 99% (root competition almost halved stem growth rate) and that for ECM was 76%, leading to a 1.3-fold difference. These differences suggest that both processes were operating more strongly between H1 and H2 than before H1.

Elemental concentrations

At H1 and H2, differences between treatments in the 10 elemental concentrations for leaf, root, and stem (N to Zn) were tested by ANOVA, simplified to a randomized block design without

Table 3. The negative effects of root competition (RCM; trmt2–trmt4) and positive effects of ectomycorrhizal networks (ECM; trmt2–trmt1) for *M. bisulcata* as inferred from differences in treatment means of shoot dry mass (g) at harvests 1 and 2 (H1 and H2), and their difference ($\Delta H = H2 - H1$), for all and allowed mesh bags.

Harvest	RCM effect		ECM effect	
	All	Allowed	All	Allowed
H1	0.027 (12.5)	0.017 (7.7)	-0.05 (-2.3)	-0.020 (-9.1)
H2	0.087 (30.4)	0.092 (32.0)	0.037 (12.9)	0.041 (14.3)
ΔH	0.060 (86)	0.075 (112)	0.042 (6.0)	0.061 (91)

Notes: Values in parentheses are differences in shoot mass expressed as percentages of the mass under treatment 2 (medium mesh) as a reference. See the main text for explanation: Shoot masses at H1 and H2 are in Tables 1 and 2.

replication for leaf, stem, and root parts at H1, and leaf at H2. Because of unequal replication in stem and root parts at H2, an unbalanced (REML) approach was used. This resulted in 60 tests: No data transformation was required. There were very few cases of significance: just one at $P \leq 0.05$, three at $P \leq 0.01$, and six at $P \leq 0.10$ (Appendix S1: Table S2): Those for Al and Fe are shown in Table 4 as they cover most of the differences of importance. (There was one other case of significance at $P \leq 0.05$, for K in roots at H2 where concentration in the coarse-mesh bags was much higher than other treatments.) At H1, Al and Fe concentrations were significantly higher, in stems of seedlings in medium-mesh bags compared with other treatments ($P \leq 0.05$). At H2, Fe in leaves was again significantly the highest under the treatment 2. And, there was a wider supporting evidence for the higher stem values across all plant parts even though significance was additionally twice at $P \leq 0.1$ only (see Table 4).

More surprisingly, there were many, often highly significant, differences between subplots (Appendix S1: Table S3), especially for Cu, Mn, and Zn in stems (more so for H1 than H2), and Cu and Zn in roots (both H1 and H2; Table 5). There appeared to be no patterns though across the elements, pairwise or for all three, in that certain subplots had associated higher or lower concentrations (Appendix S1: Table S4). Zero (undetectable) levels of Cu were recorded for two leaf samples at H1, but only in LS7; and for roots at H2, for two, two, and five samples in LS2, LS4, and LS5, where in all cases other plot replicates had very low values too. These (very-close-to) zero-values were taken as being genuine. Appendix S1: Table S4 shows the many significant differences between subplots in seedling Cu, Mn, and Zn concentrations.

Correlations between dry masses of leaves, roots, and stems, and totals, with elemental concentrations, across treatments and subplots (as were available from chemical analysis) were generally weak and insignificant at H1 (120 combinations). Just three were significant at $P \leq 0.05$ ($n = 24$), namely stem mass with P in stems ($r = -0.607$, $P = 0.002$), total mass with Mn in stems ($r = -0.486$, $P = 0.016$), and stem mass with Zn in stems ($r = -0.455$, $P = 0.026$). Eight other cases were less strongly significant at

Table 4. Mean element concentrations in the four harvest \times element combinations for which differences between treatments were significant ($P \leq 0.05$, Appendix S1: Table S2) in plant parts of *M. bisulcata* seedlings (L, leaf; S, stem; R, root) at harvests 1 and 2 (H1 and H2).

Harvest	Element	Plant part	Treatments			
			1 (0.5 μm)	2 (35 μm)	3 (250 μm)	4 (control)
H1	Al	L	431	626	461	522
		S	632 ^b	1094 ^a	650 ^b	710 ^b
		R	573	913	642	899
	Fe	L	175	244	180	214
		S	234 ^b	401 ^a	228 ^b	265 ^b
		R	181	344	203	295
H2	Al	L	296	354	213	236
		S	337	481	349	321
		R	863	805	939	771
	Fe	L	102 ^{ab}	132 ^a	61 ^b	76 ^b
		S	115	167	112	105
		R	250	239	281	237

Notes: Means that do not share any same small letters are significantly different at $P \leq 0.05$. Two other analyses H1/Fe/R and H2/Al/L were significant at $P \leq 0.10$.

Table 5. *F*-ratios for analyses of variance of mean copper, manganese, and zinc concentrations per subplot in the plant parts (L, leaf; S, stem; R, root) of seedlings of *M. bisulcata*.

Harvest	Element	Plant part		
		L	R	S
H1	df	(5,18)	(5,18)	(5,18)
	Cu	7.48***	6.35***	23.74***
	Mn	2.78*	0.17 ^{ns}	13.13***
	Zn	2.37 [§]	7.76***	6.84***
H2	df	(2,9)	(4,27)	(4,27)
	Cu	0.65 ^{ns}	46.00***	3.23*
	Mn	0.64 ^{ns}	0.85 ^{ns}	6.15***
	Zn	1.38 ^{ns}	9.26***	2.03 ^{ns}

Note: Mean concentrations per subplot are given in Appendix S1: Table S4.

*** $P \leq 0.001$; * $P \leq 0.05$; [§] $P \leq 0.10$; ^{ns} $P > 0.10$.

$P \leq 0.10$, all negative correlations again, and of these, the following are more interesting because they are stem concentrations: mass of leaves ($r = -0.362$, $P = 0.082$) and mass of roots ($r = -0.382$, $P = 0.065$) with Mn in stems.

In contrast to H1, at H2 many more, and stronger, correlations were highlighted (29 at $P \leq 0.05$). The main patterns will be mentioned. Leaf mass consistently, although in a few cases also stem mass and root mass, was negatively correlated with leaf, stem, and root N and P concentrations (five of six cases, $r = -0.379$ to

-0.690 , at $P \leq 0.05$). Calcium concentration was unrelated to any mass variable. While leaf mass showed no correlation with any K and Mg part concentration, root mass was significantly negatively correlated with root concentrations of these elements ($r = -0.426$ and -0.484 , $P = 0.015$ and 0.005) and matching stem mass with the stem concentrations ($r = -0.571$ and -0.392 , $P = 0.001$ and 0.026). Apart too from a marginally significant positive correlation between root mass and Fe in roots, stem mass was more strongly negatively correlated with Al and Fe in leaves ($r = -0.690$ and -0.642 , $P = 0.013$ and 0.025), and not in stems, more the surprising because the sample size for leaves was so small ($n = 12$). No dry mass variable was correlated with stem and root Cu, Mn, and Zn, but stem mass was negatively correlated with Mn and Zn stem concentrations ($r = -0.387$ and -0.372 , $P = 0.029$ and 0.037), once more indicating the central role of stem concentrations.

For leaf, stem, and root parts separately, and for H1 and H2 in turn, principal components analyses (PCAs, correlation matrix based) of the 10 element's variables were made. Each variable had the same subplot \times treatment/replicate structure as in the univariate analyses. PCA extracted 30–37% and 25–27% of the total variance on axes 1 and 2 at H1 and H2, respectively: Table 6 shows those variables with loadings $> |$

0.35]. Canonical variate analysis (CVA) tested for discrimination on the basis of either treatments or subplots. Treatments were generally very weakly and insignificantly separated by MVA, except for roots at H1 ($P \leq 0.05$) and at H2 ($P \leq 0.10$; Appendix S1: Table S5). But subplots were highly discriminated in all analyses except the one for leaves at H2. Multivariate analyses of variance (MANOVA) were run using subsets of just the high-loading variables from PCA (Appendix S1: Table S5). MANOVA requires that the data structure be balanced, so root and shoot variables at H2 were rebalanced by taking subplots 2, 5, and 11 only, and inserting nine “missing value” codes to give a 4 treatments \times 3 subplots \times 3 replicates structure. Only roots at H2 for the factor {Ca, K, Mg, Al, Fe} showed any difference between treatments ($P \leq 0.05$).

Forest-nursery comparison

Seedlings from the main Latin Squares experiment, those acting as unused standbys, and those remaining at the nursery will be referred to as LS, SB, and NR seedlings, respectively, from here on. At H1, the mean total mass of LS seedlings was 90% that of SB, and 22% that of NR, seedlings (Table 7). SB seedlings, however, had similar stem mass to LS ones, yet proportionally slightly more root mass; and NR seedlings had also proportionally more root mass than both types of forest seedlings. By H2, LS and SB survivors showed very similar total mass, although both were reaching only $\sim 40\%$ of what NR seedlings achieved (Table 7). All three seedling types

allocated proportionally most of their total mass to stems by H2, the LS ones the least to leaf mass. Between H1 and H2, LS seedlings declined in total mass by 20%, SB seedlings by 28%, but by strong contrast NR seedlings increased 2.4-fold. While stem mass barely altered for forest seedlings (LS and SB), for NR ones, it increased 5.6-fold. Roots of all NR seedlings were extensively well infected with ECM at H1. Furthermore, that no nursery-held seedlings (effectively saplings by H2) showed any signs of, or losses due to, pathogenic agents, above- or below-ground, demonstrates none of nursery origin affecting those seedlings planted out in the forest.

The concentrations of the macronutrients (N, P, Ca, K, and Mg) were fairly similar for LS and SB seedlings at H1. The ordering across plant parts was generally leaf $>$ stem $>$ root, with the notable exception of stem \gg leaf $>$ root for P (Fig. 3a). NR seedlings had slightly lower concentration of macronutrients than forest (LS and SB) seedlings at H1, especially for stem and root, sometimes for leaf (Mg), although again one exception was the far higher concentrations of P in LS and SB seedlings than the NR ones (~ 3 -fold more in stems), and the second conversely much higher leaf, and moderately higher stem and root, concentrations of Ca in NR than LS and SB seedlings (Fig. 3a). At H2, the macronutrient concentrations were similar for seedling types and plant parts to those at H1, with the notable exception of a marked higher concentration of K in the leaves of NR than LS and SB seedlings (Fig. 3b).

Table 6. Principal components analysis of 10 elemental concentration variables in plant parts of *M. bisculcata* seedlings (L, leaf; S, stem; R, root) at harvests 1 and 2 (H1 and H2), indicating the variances accounted for by the first three axes, and those variables with loadings $\geq |0.35|$ (superscripted + and -).

Harvest	Plant part	n	% variance on axes			Loadings on axes	
			1	2	3	Factor 1	Factor 2
H1	L	24	29.5	24.9	18.5	⁺ Ca, ⁺ Al, ⁺ Fe, ⁺ Mn	⁺ N, ⁺ P, ⁺ Zn
	S	24	34.9	27.4	14.0	⁺ Al, ⁺ Fe, ⁺ Cu	⁺ P, ⁺ K, ⁺ Mn, ⁺ Zn
	R	24	36.7	24.8	11.7	⁺ N, ⁺ Al, ⁺ Fe, ⁺ Mn, ⁺ Zn	⁺ N, ⁺ Ca, ⁺ K, ⁺ Cu
H2	L	12	37.2	25.2	18.7	⁻ N, ⁺ Al, ⁺ Fe, ⁺ Mn	⁻ K \blacktriangleright F3: ⁻ P, ⁻ Mg, ⁻ Cu
	S†	32	31.1	26.6	12.1	⁺ N, ⁺ P, ⁺ Al, ⁺ Fe, ⁺ Cu	⁻ P, ⁻ K, ⁻ Mg, ⁺ Al, ⁺ Fe
	R†	32	30.5	25.5	11.2	⁻ Ca, ⁻ K, ⁻ Mg, ⁺ Al, ⁺ Fe	⁺ N, ⁺ P, ⁺ Cu

Note: A third factor was used instead of the second in the arrowed case and for H2 a rebalanced data set (see text) for S and R.

† Alternative PCA with rebalanced data for the MANOVA in Appendix S1: Table S5; variances S, 34.9, 27.6, and 11.5%; R, 32.8, 24.2, and 12.3%.

Table 7. Means and SEs of dry masses (g) of plant parts (L, leaf; S, stem; R, root), and their totals (T) achieved by seedlings of *M. bisulcata* by harvests 1 and 2 (H1 and H2), with sample sizes (*n*), when transplanted into the forest experimental Latin Squares (LS), maintained as standbys in the forest (SB), and left to grow in the nursery (NR).

Harvest	Plant part		LS	SB	NR
H1		<i>n</i>	74	15	18
	L		0.205 ± 0.010	0.255 ± 0.017	0.878 ± 0.112
	S†		0.231 ± 0.007	0.226 ± 0.013	0.849 ± 0.079
	R†		0.169 ± 0.008	0.191 ± 0.014	0.962 ± 0.134
	T		0.605 ± 0.021	0.672 ± 0.028	2.690 ± 0.313
H2		<i>n</i>	32	10	7
	L		0.095 ± 0.009	0.141 ± 0.025	3.594 ± 0.442
	S		0.231 ± 0.013	0.201 ± 0.016	4.721 ± 0.842
	R		0.158 ± 0.025	0.140 ± 0.013	2.893 ± 0.666
	T		0.483 ± 0.025	0.482 ± 0.043	11.207 ± 1.545

† Including seedlings leafless and recently dead (*n* = 85) stem and root masses 0.216 ± 0.008 and 0.158 ± 0.008, respectively.

The other five elements measured can be divided into the non-nutritional one Al, two mesonutrients Fe and Mn, and then two micronutrients Cu and Zn. Apart from LS seedlings at H1, Al concentrations were otherwise higher in roots than leaves and stems at both harvests, more so for H2 than H1 though (Fig. 4a, b). Higher concentrations of Fe were found in roots than leaves and stems of LS seedlings at H2 but not at H1, and likewise for SB seedlings at both harvests, while NR seedlings lower in Fe concentrations showed small differences between plant parts (Fig. 4a). Patterns across plant parts were similar for H1 and H2, with the ordering leaf > stem > root for LS and SB seedlings, leaves still much higher than stems and roots for NR seedlings. For the micronutrients, Zn varied rather little between seedling types and plant parts, within and between harvests, except the decreasing concentration in all parts between H1 and H2 for NR seedlings (Fig. 4a, b).

Nursery seedlings had high concentrations of Cu at H1, especially in the leaves, and with ordering leaf > stem > root (Fig. 4a), but by H2, they had fallen to less than a tenth, with much lower concentrations in stems, an ordering stem << leaf ~ root (Fig. 4b). Field seedlings (LS and SB) had more Cu in roots than leaves and stems (Fig. 4a), and then by H2, it remained very low with approximately similar concentrations in all plant parts, much lower in roots and shoots

than at H1, but with only a corresponding slight reduction in leaves (Fig. 4b). As with the main plot experiment, a few zero Cu concentrations occurred among the standbys, one a leaf sample at H1, in again LS7; and for three root samples at H2 but here in LS2 (once) and 9 (twice). Accompanying values for the same plots were also very low. These (close-to) zero-values were therefore taken as being valid for the comparison of the three seedling types.

Soil chemistry

The later soil sampling was an attempt to explain the large differences in elemental concentrations between subplots. Copper concentrations were zero across all samples, or at least were so very low as to be undetectable. This is a remarkable finding. Cobalt concentrations were similarly near zero at the lowest depth, and very low at the top and middle ones (Table 8). The other four elements, Al, Fe, Mn, and Zn, had appreciable levels, the latter three of which declined steeply with depth over the 10-cm sampled. These four, at each depth, and averaged over the three depths, were correlated with the same elements in leaf, stem, and root at H1 and H2 (subplot-level means) and their means-of-parts (plant-level) concentrations, and with those of Cu in the plant parts too. Further, Al and Fe soil concentrations were cross-correlated with L, S, and R plant concentrations of Mn, Cu, and Zn, and Mn with Cu and Zn, yet resulting overall in

(a)

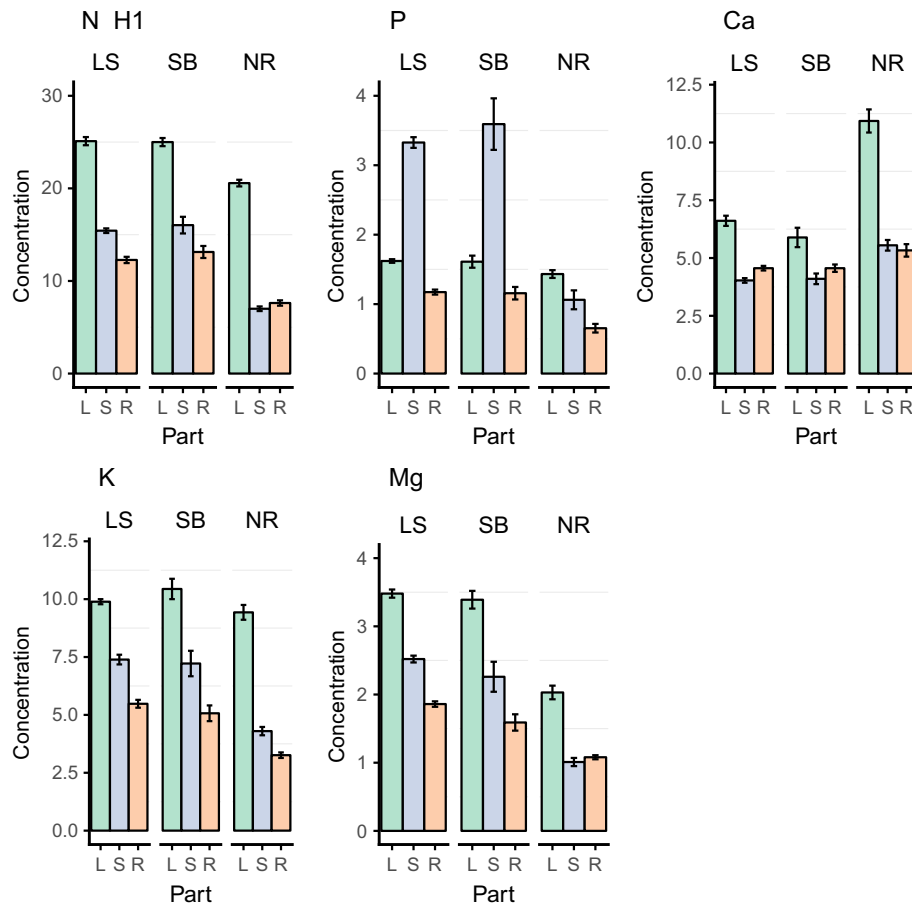


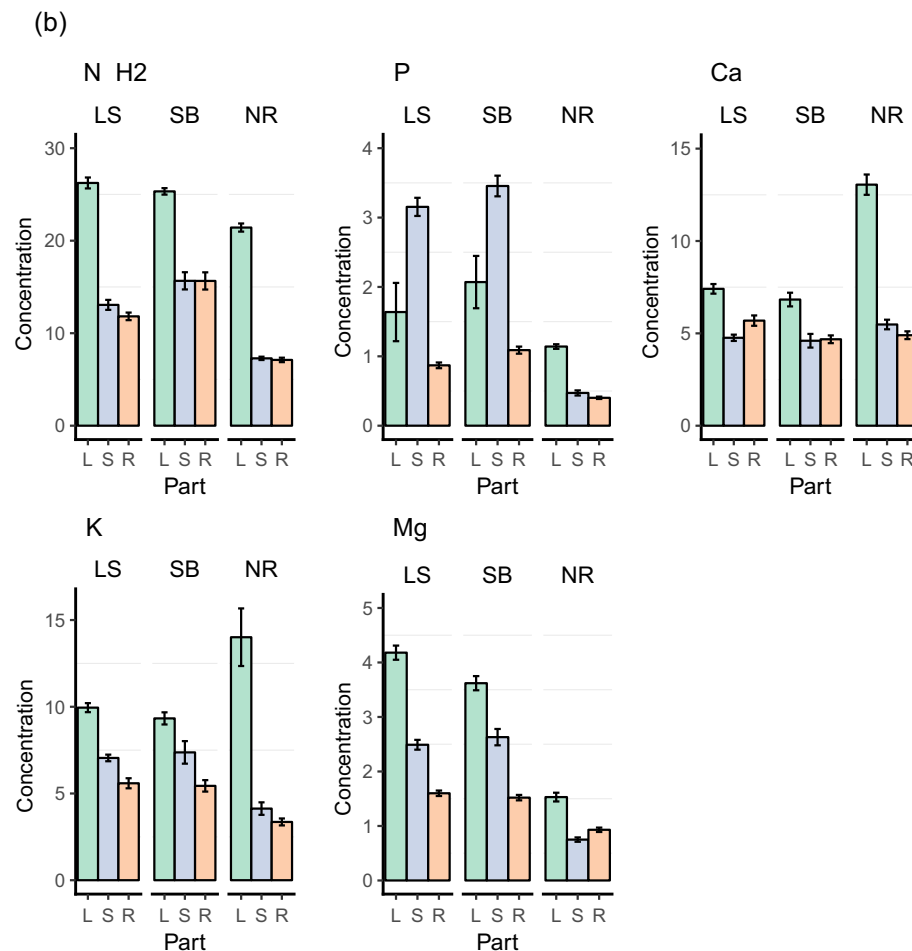
Fig. 3. Mean concentrations of nitrogen, phosphorus, calcium, potassium, and magnesium (mg/g) in the plant parts: leaf (L), stem (S), and root (R) of seedlings of *M. bisulcata* surviving to (a) harvest 1 (H1) and (b) harvest 2 (H2), which were grown in the Latin Square experimental subplots in the forest (LS; means of four treatments), as standby plants in the forest (SB), and remaining at the nursery outside the forest (NR). Values for LS and SB seedlings are also averaged over six subplots per harvest; the NR ones are means of replicates in two nursery blocks. Error bars are ± 1 SE.

only very few scattered cases of significance ($P < 0.05$).

The two reportable sets of consistent and significant correlations, however, were for Mn at H1 being, respectively, positively and negatively related to Al concentrations in the top and middle layers for roots ($r = 0.955$ and 0.941 , $P < 0.02$) and the lowest layer for shoots ($r = -0.988$, $P = 0.002$); and for Zn at H2 positively related to Fe concentration in the middle layer for roots and shoots ($r = 0.944$ and 0.961 , $P < 0.02$). Otherwise, there was no particularly

convincing explanation for the variation in plant Cu, Mn, and Zn concentrations across the plot, apart from the negative correlation between Cu in leaves at H1 with Al soil concentration at the lowest depth ($r = -0.914$, $P = 0.030$).

Mean inverse-distance weighted basal area estimates (wba, dm^2/m) of the two trees next to each subplot (Appendix S1: Table S1) were not correlated with either soil or seedling Al–Zn concentrations, except for soil Mn at 6–10 cm ($r = 0.638$, $P < 0.05$) and soil Zn at 2–6 cm ($r = 0.663$, $P < 0.02$). There was therefore very

(Fig. 3. *Continued*)

little evidence that neighbor *M. bisulcata* effects might be simply related to these seedling elemental concentrations.

DISCUSSION

Evidence for ECM linkages

Seedling height, leaf number, and survival rate were very similar to those found in previous studies of *Microberlinia bisulcata* seedlings at Korup, suggesting that growth and dynamics were representative of natural populations and comparable to other experiments (Newbery et al. 2000, 2002, 2004, 2010, Green and Newbery 2001b, 2002). The blocks and subplots were of a suitable design to cater for the natural spatial heterogeneity of the forest grove, with view to

allowing a high accuracy of treatment comparisons, which was indeed improved by using covariates. Root penetration of the mesh bags was an unforeseen technical problem but allowing for it (i.e., discounting those cases so that only intact bags were considered) did not alter the main experimental conclusions. Some roots, but not all, came from cut ends when setting in the bags. Making holes in the ground substantially larger than the bags would have meant even larger distances between seedlings and the surrounding forest floor root mat, thus further diminishing the possibility of detecting treatment effects. A major operational problem for all experiments using meshes of successive size in the field, to exclude hyphae and roots, is that they do not allow for a treatment roots without

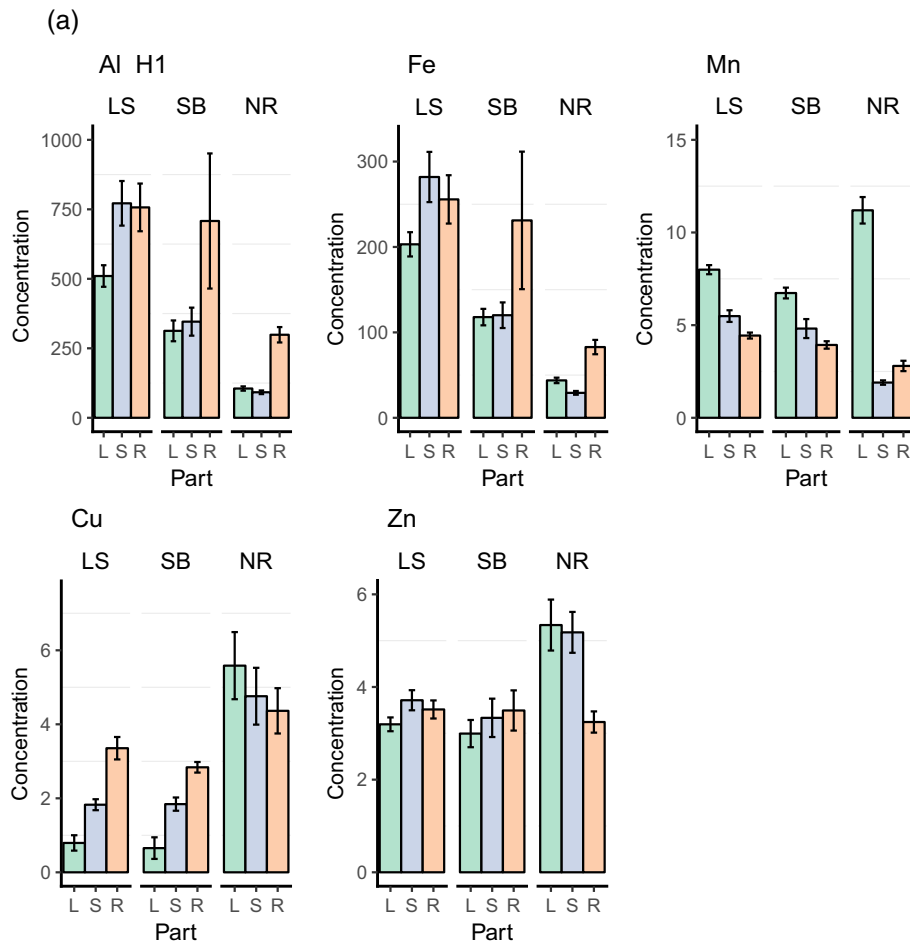
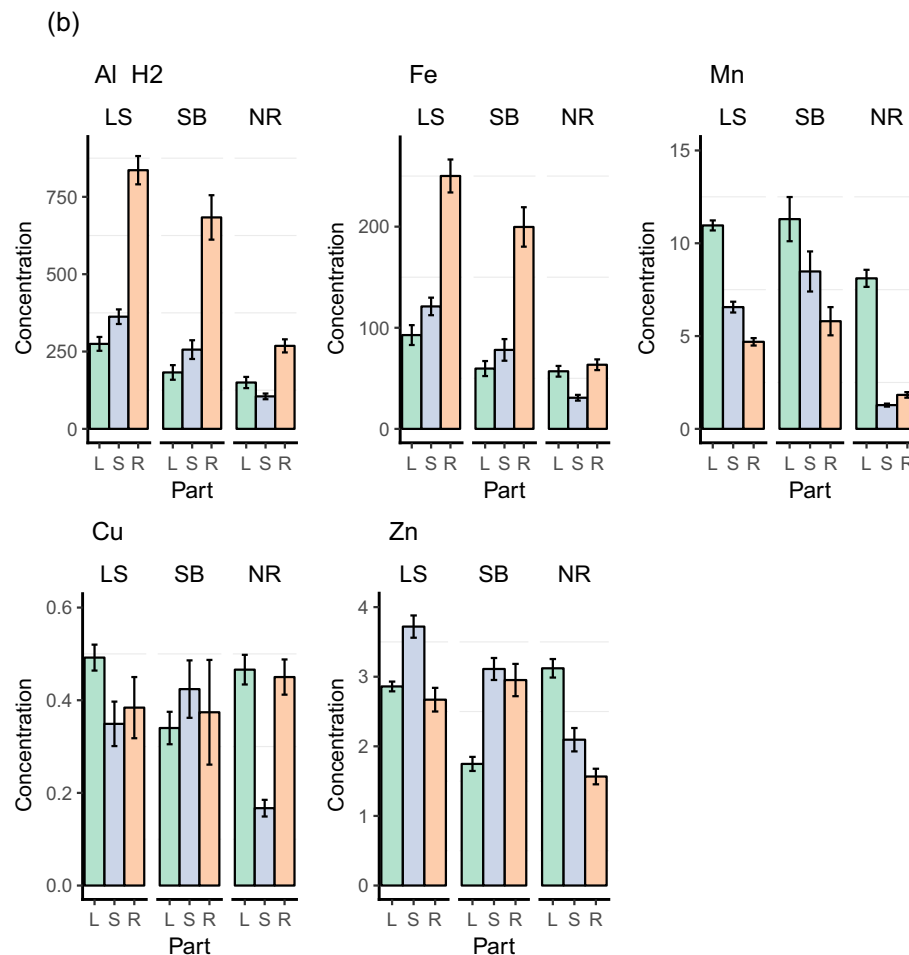


Fig. 4. Mean concentrations of aluminum, iron, manganese, copper, and zinc (mg/100 g) in the plant parts: leaf (L), stem (S), and root (R) of seedlings of *M. bisulcata* surviving to (a) harvest 1 (H1) and (b) harvest 2 (H2), which were grown in the Latin Square experimental subplots in the forest (LS; means of four treatments), as standby plants in the forest (SB), and remaining at the nursery outside the forest (NR). Values for LS and SB seedlings are also averaged over six subplots per harvest; the NR ones are means of replicates in two nursery blocks. Error bars are ± 1 SE.

ECM, and therefore, no exact factorial decomposition of the two main effects is possible.

Differences between treatments were small and few, mostly non-significant ($P > 0.05$). At H1, leaf (and total) mass did show significant differences between treatments ($P < 0.025$), and at H2, stem mass was marginally significant ($P < 0.055$). However, these differences were not as large as was predicted, namely that preventing ECM linkages (fine-mesh treatment) between adult *M. bisulcata* trees and seedlings would clearly reduce the latter's growth and survival. In

plant growth studies, leaf and root mass measured at a harvest is the outcome of organ turnover, while stem mass is a permanent accumulation. Allowing for root penetration weakened the apparent effects for leaf and root mass at H1 but it strengthened them for stem mass at H2. Differences in stem mass between treatments and over time provided therefore the most robust results on several counts. There also remains the more limited alternative hypothesis that ECM networks may be transferring carbon and mineral nutrients to be put mostly into

(Fig. 4. *Continued*)

seedling stem growth, with little immediate effect for leaves and roots.

ECM scoring also produced unexpected results. The three mesh sizes did not lead to large differences in the level of root infection (28–46%): It was indeed highest for the mesh size that aimed to exclude ECMs the most. By contrast, the control had the least infection (5%), where seedlings were completely open to outside root and ECM contacts. ECM infection was also not associated with root penetration. Assuming that all of the transplanted seedlings from the nursery were infected, mesh bags were apparently affording some form of protection to the ECMs, and those in the medium (all and allowed bags), and possibly also coarse-mesh bags (allowed), some growth promotion. At either end of the mesh size gradient (fine mesh vs. control),

different factors likely operated to limit growth. Further, it would seem that benefits to growth were only possible when ECMs were accompanied by their finest roots; in other words, connections to the seedlings were possible only when roots carried the advancing hyphae or permitted rhizomorphs—within the time and space limitations of the experiment. Compared with what was achieved on average in the coarse-mesh bags and controls, medium bags and fine bags led to increases in 40 and 22% in stem mass, respectively. That the increase in the latter treatment is less than the former fits with the time-distance advancement explanation.

Between H1 and H2 effects switched from leaf to stem mass, with leaves (in terms of mass and number) declining, root (mass and length) being static, and stem (mass and height) increasing,

Table 8. Extractable concentrations of six elements (means \pm SE; $\mu\text{g/g}$) at three depth ranges in the soils adjacent to experimental subplots ($n = 11$; values for LS3 were not achievable).

Depth (cm)	Al	Fe	Mn	Zn	Cu	Co
0–2	237.0 \pm 36.8	9.79 \pm 1.18	5.73 \pm 0.62	1.246 \pm 0.123	0.0	0.009 \pm 0.004
2–6	234.4 \pm 15.8	4.92 \pm 0.31	1.14 \pm 0.11	0.470 \pm 0.044	0.0	0.001 \pm 0.001
6–10	213.2 \pm 10.5	2.63 \pm 0.26	0.94 \pm 0.20	0.436 \pm 0.092	0.0	0.0

with the net effect of total mass declining. What gave advantages to leaf production by H1 (medium-mesh bags) was evidently lost or was ineffective by H2. Clearly, in the field, after transplanting, roots were making poor progress (Appendix S2), stunted with very few laterals, not growing, and therefore little more advanced than they were in the nursery, possibly even degenerating. A key process was root establishment and growth. There could have been two causes: (1) Low-light levels in the forest meant a carbon deficit (at least up until H1) and hence very little allocation to root growth was possible; or (2) soil factors may have inhibited rooting and nutrient uptake and then, as a consequence, leaf growth. The standby seedlings fared no better than the experimental ones, and they did not have their roots in direct, embedded, contact with the soil. This might argue for light limitation being the main determinant of the experiment's outcome. Non-destructive measurements support the dry mass data and, even while they were more frequently recorded, they do not give any clear clues that could explain the variation in leaf numbers, for instance. They do confirm though the typically very slow and limited height growth of all experimental seedlings in these groves, as in previous studies.

All seedlings within bags could have been re-inoculated by mycorrhizal spores, but presumably to the same extent for each treatment. The mesh design also did not stop hyphae potentially crossing over rims to get inside bags, perhaps aided by fallen decomposing leaves or twigs affording bridges from outside (Fig. 1). It was just not feasible to clean each bag's soil surface regularly, even if that had been seen as appropriate.

Role of light and root competition

Seedlings remaining in the nursery provided another valuable comparison. They were of the same seed source, age, and initial handling; the

principal difference from the forest being their better-lighted growth conditions. Seedlings grew much better in the nursery, having especially well-developed, highly ECM-infected, root systems; and they showed substantial increases in stem mass and height between H1 and H2. This matches what is well established about *M. bisulcata* being light-demanding and strongly shade-intolerant when very young (Green and Newbery 2001a, b; Newbery et al. 2006b, 2010). That said, the present experiment was done in the forest shade to specifically test whether adults could affect seedling survival and growth through ECM linkages, in the hope of explaining the species' very poor in situ recruitment. Doubtless, had a similar experiment been conducted in the nursery with seedlings next to large saplings or small plantation trees, or with seedlings within canopy gaps in the forest, the results would have been quite different. In all likelihood, the finest mesh treatments would have shown more reduced seedling growth. But that would have obviated the problem of understanding the low survival of seedlings in shaded understorey of closed-canopy forest groves.

The low-light conditions in the forest most likely therefore limited seedling growth which led to poor root extension and hence severely limited the efficacy of the treatments because in the time span they largely would not have been able to make strong ECM-alone connections. The results point to this operating when fine- or medium-sized roots were allowed to enter the bags. Any ECM connections made were evidently also not sufficient to tip the C-balance enough in their favor to increase seedling establishment and survival. Proximity to adult *M. bisulcata* trees was not found to promote seedling survival in several earlier studies at Korup (Newbery et al. 1998, 2000, 2006b, 2010, Norghauer and Newbery 2016). However, studies at other, mostly temperate, sites, investigating the role of ECMs have

indicated that it is under such shaded conditions that ECMs might be needed to help seedlings survive better (see *Introduction*).

Given the root penetration results, it is highly likely that outside roots did reach most seedlings in the medium- and coarse-mesh bag and control treatments. A large majority of these roots would certainly have come from the large *M. bisulcata* trees surrounding the LS subplots because the grove is dominated by them and, moreover, the four blocks worked in were selected intentionally because they had the highest basal areas of the species within the P-plot (Neba 2015).

Mesh bags will also minimize the effect of root competition, perhaps not completely as roots pressing up against the sides of a bag from the outside could draw nutrients from the soil within. Thus, the fine and medium meshes can be seen as preventing intermingling and root competition close to seedlings. Coarse mesh and controls would have afforded moderate and full competition, respectively. It is valuable to note that at H2, shoot dry mass with allowed was greater than with all bags, when bags with rootpen2 = 2.5 values (i.e., heavy root penetration) were excluded, lending support to the notion that root competition was a major factor under this treatment, and accordingly the smaller meshes limited its influence.

To speculate on how ECMs could be of benefit to juvenile *M. bisulcata*, it appears they must first be well established with a good root system and ECMs, and would then also require bouts of high light conditions. Seedlings or saplings are then in a better state to make linkages and benefit from any nutrient transfers. Seedlings need to be primed in their nutrient reserves, that is, they must be well stocked. As Newbery et al. (2006b) clearly showed with naturally established cohorts, being close to adults was of no particular benefit to *M. bisulcata* seedlings, indeed the converse. When seedlings much larger than those used in this experiment, and their roots had been sterilized before transplanting, they became infected with mycorrhizas in stands of both high and very low basal area of ECM caesalp species (Newbery et al. 2000). Norghauer and Newbery (2016) found that naturally regenerating *M. bisulcata* seedlings showed first positive density dependence on adult tree basal area, and this switched to negative density dependence over

about 4 yr: Being close to adults slowly disadvantaged seedlings with time. As suggested in Newbery et al. (2010), the seedling or sapling that is well-established benefits from bouts of higher light levels, growing up in a ratchet-type manner. Between such micro-events, the seedling must survive many months or even years in moderate-to-deep shade, and it is perhaps then that ECM connections would be expected to be beneficial in maintaining the sapling above its compensation point.

An alternative thesis for the poor survival close to adults was that through the ECM linkages the flow of carbon was reversed, not from adult to seedling, but the other way, based on the argument that the old very large grove trees would have high respiration loads and therefore create a sink for the seedlings resources. This would imply that under those conditions seedlings would suffer being close to parent trees (Newbery et al. 1998, 2000). That bidirectional flow might operate could then nullify seedling support or lead to an intrinsic instability or alternation of flows in two directions, driven entirely by source-sink concentration gradients fluctuating in the ecosystem. Other factors would influence the flows, and it is unknown whether C or P, or another element like Cu, would be the resource most in demand.

In the latter context, *M. bisulcata* had moderate-to-high mast fruiting in southern Korup in 2013 and 2014 (*unpublished data*), an unusual double-year of fruiting similar to 1997 and 1998 (Newbery et al. 2006a). In May 2014 and November 2015 (at H1 and H2), many pod valves from 2013 to 2014, respectively, were lying in and around the LS subplots (Fig. 1). This may well have exerted a considerable sink in the adult trees, as hypothesized by Newbery et al. (1998) and Norghauer and Newbery (2016).

Involvement of micronutrients

The efficacy of the ECM symbiosis might lie in part in the nature of the soils and nutrient supplies at Korup. The soils are highly sandy, of low pH, well-draining, and nutrient-poor, especially in P and K (Gartlan et al. 1986, Newbery et al. 1997). In the present experiment, differences between treatments in elemental concentrations were rather few and consequently only weakly significant. There was also some indication of

Mn in stems being affected by treatments. Correlations between concentrations in the different plant parts by H1 were mostly also weak, suggesting high instability in stoichiometric patterns of nutrient allocation. By H2, though, when some growth had been made by survivors, N and P concentrations (especially the latter) had increased in smaller seedlings' leaves, stems, and roots, and K and Mg in just roots. Conversely, larger seedlings had more Al and Fe in their leaves, less Mn and Zn in their stems, but Cu remained unrelated to plant part mass. As growth slowed essential macronutrients were probably being recycled into any remaining, or occasionally new, leaves on the still small seedlings; and Al and Mn concentrations were declining due to generally restricted uptake caused by poor root growth.

The nutrient concentrations, especially those in leaves, indicated that LS seedlings had 8 mg/kg Cu at H1, but this fell to 5 mg/kg at H2. Zn concentration was ~30 (32, 29) mg/kg at both H1 and H2; Mn concentrations ~80 and 110 mg/kg correspondingly. Comparing with compendia for agricultural crops (Jones 1991, Kopsell and Kopsell 2007, Marschner 2012, Yruea 2015), Cu at H1 was of normal concentration (in the 5–30 mg/kg range), but at H2, it had become critically low or just deficient (by these norms, 2–5 mg/kg). Drechsel and Zech (1991) in their synthesis of concentration in leaves of tropical trees also place values of 2–5 mg/kg at low-to-deficient, and normal ones at 5–30 mg/kg. In crops, Zn commonly becomes deficient at 10–20 mg/kg, the normal range being 20–150 mg/kg; For tropical trees, the ranges inferred are 3–10 and 10 to ~40 mg/kg, respectively. Zinc concentrations in the LS seedlings were therefore probably sufficient for growth, those for Cu not, by H2. Turning to Mn, 15–20 mg/kg was considered deficient for crops and 20–300 mg/kg normal, the corresponding ranges for tropical trees being 10–30 and 30–200 mg/kg. Mn at H1 and H2 was thus sufficient, if not on the high side by these norms. Put alongside the changes in Zn and Mn between H1 and H2, Cu was becoming rapidly deficient in seedlings leaves. LS and SB seedlings had similar concentration of these three elements in leaves, and also for stems and roots, even though the latter were generally much higher.

Particularly interesting are the relative differences in concentrations in the plant parts between H1 and H2: At H1, stems, and more so roots, had higher concentrations of Cu than leaves, but by H2, these parts had very similar concentrations. Presumably, the non-balancing higher Cu that was held in roots by H1 was transferred to the leaves, via stems, so that the, albeit, much-reduced concentrations at H2 were about even. By contrast, seedlings maintained similar Zn and Mn proportions in their parts' concentrations between H1 and H2. Finally, as a further contrast, Fe concentrations in leaves at H1 were ~2030 mg/kg, changing to ~930 mg/kg at H2, which by crop norms would imply toxic levels (defined as > 500 mg/kg, Jones 1991), and by tropical tree norms they would be ranked very high (higher than any values tabulated by Drechsel and Zech 1991). Leaf concentrations of Al changed from ~5100 to ~2750 mg/kg between H1 and H2, which might indicate accumulation in the seedlings (Drechsel and Zech 1991, Miyasaka et al. 2015): Concentrations in stems and roots were much higher.

Aluminum and Fe concentrations were much higher under treatment 2, the medium-mesh bags, than under the other treatments, and this treatment had the highest shoot dry masses. Better growth was therefore associated with uptake of more Al and Fe, averaged over the LS subplot values: Overall mass vs. concentration correlations supported negative trend between Al and Fe in leaves with stem mass. Apparently, *M. bisulcata* can accumulate Al, mainly in the roots; the Fe levels are indicative of toxicity, however. For this treatment, neither stem P concentrations nor ECM scores were the highest, though. The medium mesh allowed ECM hyphae to pass but not roots: The results in this aspect remain paradoxical, and unresolvable given the considerably complicated nature of the interactions between soil, light level, and treatments, and the various nutritional, mycorrhizal, and seedling growth processes.

In contrast to the weak differences among the treatments within the subplots in the forest, differences between plots, averaged across treatments, were much stronger. Notably, variation in dry mass of roots was larger than that of stems or leaves across subplots. Heterogeneity in soil elemental concentrations was also large, and

even if the LS design did cater for variation locally (over a scale of 4–5 m), there was far more variation between LS locations than between treatment replicates. Especially evident were the significant differences between subplots in Cu and Zn concentrations for leaves, stems, and roots at H1, and for roots at H2; plus differences in Mn concentrations for stems at H1 and H2. It seems that seedlings were sensitive and responsive to critically low Cu and low Zn sources. If another main element like K or Mg were limiting growth and Cu and Zn were in sufficient supply, then such differences would not be expected. Once more, it is found that the most variation in nutrient concentrations among subplots (including the very high P concentrations remarked on before) is in the stems. In the PCAs, P and Zn (two cases of 12) and P and Cu (three cases) were both positively loaded as variable pairs, but for two factors of the six different harvest \times plant part combinations: Cu and Zn were not together correlated with P on any axis suggesting disparate processes at work. Any adult-mediated ECM network treatment effect was really quite minimal compared with this forest spatial variability, in its potential effects on seedling nutrient concentrations. Connected to this, it was root mass that was significantly varying the most between subplots among the plant parts.

While Cu and Zn are mostly in solution in mineral soils below pH 5, when the latter are sandy and heavily weathered, as at Korup, concentrations can be critically low (Harter 1991). Furthermore, in especially tropical soils with high levels of Al and Fe, Cu will be complexed with these elements, and they will also have adverse effects on Cu uptake by plants. Combined with this is the well-established result that Cu becomes strongly adsorbed on organic matter (Stevenson 1991), and in the case again of Korup, in that critical surface organic layer and root mat overlying the sandy soil. Vesicular arbuscular mycorrhizas (VAM) on crops have been shown to aid roots in acquiring Cu and Zn, in a way similar to the one they do for P (Marschner and Dell 1994), because Cu especially, more than Zn, is very slow-moving in the soil. There is very limited evidence that ECMs might have a similar role, and even store Cu (Marschner 1995). Often shown for VAMs on crop plants, and a few cases for ECMs on trees, is that when P is added as a

fertilizer, it can create Cu and Zn deficiencies. With Al toxicity affecting Mg supply, which affects fine-root growth (see Neba et al. 2016 in this context), and extra P suppressing ECM development further, all these factors appear to conspire to limit Cu availability and uptake. The seedlings in this experiment had accumulated P indicating that relative to other elements there was a luxury concentration in the plants, and this may further imply that P was not encouraging ECMs on the roots either.

Copper is reported to move slowly within plants (Kopsell and Kopsell 2007), and if taken up tends to reside in the roots first (and possibly in ECMs). George et al. (1994) have shown that when P supply is increased, albeit for crop plants with VAMs, Cu is shifted progressively from root, to stems, then leaves. Indeed, in this study, at H1 Cu was much higher in roots, than stems and lastly leaves, although by H2 these had equalized at much lower concentrations. ECMs might be able to protect roots from free Al^{3+} ions that are toxic (Smith and Read 2008, Marschner 2012). This was partly confirmed by the strong negative correlation of Cu in leaves at H1 and soil Al concentration. In this experimental situation at Korup, highly unstable dynamics were probably operating inside the rhizosphere domain, and the inability for roots to grow and form extensive ECMs worsens the seedlings' potential to take up micronutrients, especially Cu. Zinc, being held less firmly on organic matter, more mobile in soil solution, indeed made more available in soil as pH decreases, and apparently less affected by Al and Fe, means that it will also be less limiting to seedling growth, as the results here show.

It is noteworthy that between the second and third editions of both *Mycorrhizal Symbiosis* (Smith and Read 1997, 2008) and *Mineral Nutrition of Higher Plants* (Marschner 1995, 2012), there has been very little progress to report on the role of mycorrhizas in micronutrient, namely Cu and Zn, uptake by plants, and particularly lacking is work on ECMs of trees. Given that Cu deficiency is of worldwide concern for crops on nutrient-poor, acidic, and weathered tropical soils, it would be surprising that this element did not play a key role in controlling nutrient cycling in natural forests under these conditions.

The soil analyses showed again how critically low were the Cu concentrations at Korup. The

values were from a KCl extractions: A stronger reagent may possibly have removed a little more Cu. It is difficult to know exactly how representative an extract will be of the element's availability in any case. Since Cu is crucial for fungal and tree physiologies (Marschner 1995), and hence ECMs, then on the one hand, ECMs should when they operate aid Cu acquisition, but on the other they will depend on it strongly to get started and grow. In the ECM-dominated groves at Korup, it appears that the *M. bisulcata* and *Tetraberlinia* spp. trees reduce the Cu concentrations in the soil to very low levels, in a substrate that is already inherently deficient, and considerable selection pressure would be expected for traits to conserve this micronutrient within the living biomass. Perhaps the key limiting element in *mature* groves is Cu, and not P? Phosphorus availability may be the leading factor early on in the cycle of grove dynamics (Newbery et al. 2013) but as the stands mature, and P-cycling is operating efficiently (Chuyong et al. 2000), there could be a premium placed on Cu availability. This might also explain why the treatments affecting potential ECM linkages did not fully work if the seedlings were Cu-limited; and it would also be an answer to why, in the P-fertilization trial close to Korup, P-limitation could not be demonstrated and another limitation was indicated (Newbery et al. 2002). Conversely, in applying a Cu-based fungicide to test for leaf pathogens, Norghauer et al. (2010) did not see any growth improvement in *M. bisulcata* wildings, and the leaves had high P concentrations: It is not known whether the Cu applied was available for growth, however.

Comparing field seedling (LS and SB) with those of the nursery (NR), there were small differences N, Ca, K, and Mg macronutrient concentrations, far fewer than expected given the much larger masses attained by the nursery seedlings, but the much higher concentrations of P in the forest than the nursery are striking, over threefold at H1 and over fivefold at H2. A P-accumulation effect was found in other earlier studies, indicating sufficient P being taken up by seedlings and suggestive of a limitation by another factor (Green and Newbery 2001b). And, while concentrations in Al, Fe, Mn, and Zn were quite similar, it was for Cu that again marked differences occurred. In the nursery, the concentration of Cu was adequate

at least in the leaves, but by H2, they had been reduced to levels similar to the forest seedlings, presumably as a result of dilution effects of growth. As a result, Cu could also become critical for these seedlings despite their head start in height, root mass, and ECM development because they then started to rely more on the underlying old oil palm soils in the nursery. That Cu was also very low in the plot soils, and highly variable subplot to subplot (unrelated to neighboring *M. bisulcata* basal area), suggests that its availability could have been low to near zero as a result of local topography, soil chemistry, and possibly also tree demand.

The influence of systemic pathogens on *M. bisulcata* seedling growth and survival in this particular experiment cannot be completely ruled out. A fungicide treatment would likely have affected the mycorrhizas as well though. Nevertheless, it is cautiously recognized that further research on possible pathogens of the caesalp species at Korup is required (see Norghauer et al. 2010).

CONCLUSIONS

The experiment at Korup demonstrated that root competition is likely an important factor that negatively affects the growth of *M. bisulcata* seedlings, and that it cancels and overrides the smaller positive effects that apparently occur through seedlings being able to access the ECM network at Korup. However, low-light levels, together with high Al and Fe, and critically low Cu, concentrations in the soil, prevent seedlings from growing adequately during the early stages of establishment and, therefore, adequately reaching the network. These two sets of factors compound one another: Together, they reasonably explain the very low recruitment rates of *M. bisulcata* observed in the forest. Soil Cu concentrations might be driving root competition in this ecosystem. Nevertheless, for a seedling to acquire sufficient Cu would mean that it must already have extensive fine roots and ECMs—unless, of course, the Cu is being transferred to it through the network from the adults, which seems unlikely as the adult trees also require much Cu. Making fully functional connections via ectomycorrhizal hyphae in a network does not seem possible for this species' seedlings when

they are growing under shaded conditions and in such impoverished soils.

The results here for *M. bisulcata* do support those of two other tropical studies, on *Dicymbe* (McGuire 2007) and *Paraberlinia* (Onguene and Kuyper 2002), which also involved ectomycorrhizal caesalpiniaceous tree species and inferred that ECM networks were likely assisting growth and survival of conspecific seedlings close to adults. The effect of ectomycorrhizas for *M. bisulcata* was, however, quite small and approximately half the estimated countering effect of root competition. The latter process surely operated in the other studies as well. Evidence from the field for the transfer of nutrients between adult trees and their seedlings via ECM networks remains, unfortunately, far too incomplete to reach any conclusion as yet about its importance for the dynamics of tropical rain forest ecosystems.

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DATA AVAILABILITY

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.5qc1jc3>.

SUPPORTING INFORMATION

Additional Supporting Information may be found online at: <http://onlinelibrary.wiley.com/doi/10.1002/ecs2.2686/full>